Collagen Fibrils in DDR1 Deficient Mice Modulate Platelet Adhesion

THESIS

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By

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Abstract

Platelet adhesion to the sub-endothelial collagen is primarily mediated through the binding of platelet receptors, such as glycoprotein VI (GPVI) to collagen and GPIb to collagen-bound von Willebrand factor (VWF). The role of non-platelet derived receptors in modulating platelet-collagen interaction is not well-understood. Discoidin domain receptor 1 (DDR1) is a collagen-binding receptor tyrosine kinase that inhibits collagen fibril formation and disrupts the native banded structure of collagen fibers. We have recently shown how the adventitia of DDR1 KO mice exhibited collagen fibrils with larger diameters, and an increase in the depth of D-periods as compared to their wild type (WT) littermates. The purpose of this study was to examine if changes in the collagen fibril structure in the DDR1 KO vessel wall impact platelet adhesion and the extent to which this is modulated by VWF vs. GPVI. Human platelet-rich plasma was incubated, both with and without VWF or GPVI inhibitors, over aortic cross sections from DDR1 KO and WT mice under static conditions. Platelet adhesion to the adventitia of the vessel wall was evaluated using indirect immunofluorescence microscopy. Quantitative analysis of platelet adhesion to the adventitia was carried out by analyzing the area of platelet particles per unit area of collagen. The results displayed that DDR1 KO mice had greater platelet adhesion to adventitia than WT. Also, DDR1 knockout mice showed greater inhibition than the WT mice in the presence of VWF inhibitors. We thus elucidate that changes in collagen fibril ultrastructure impact platelet-collagen adhesion by altering the number of GPVI binding sites available on collagen fibrils. This knowledge is important for pathological conditions, such as atherosclerosis and aneurysms, where collagen is extensively remodeled, and could lead to altered collagen fibril structure and thrombogenic events.

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Additionally, I would like to thank Jeff Tonniges for his mentorship and aid throughout this project, as well as Mark Ruegsegger for being on my thesis defense committee and his helpful comments.

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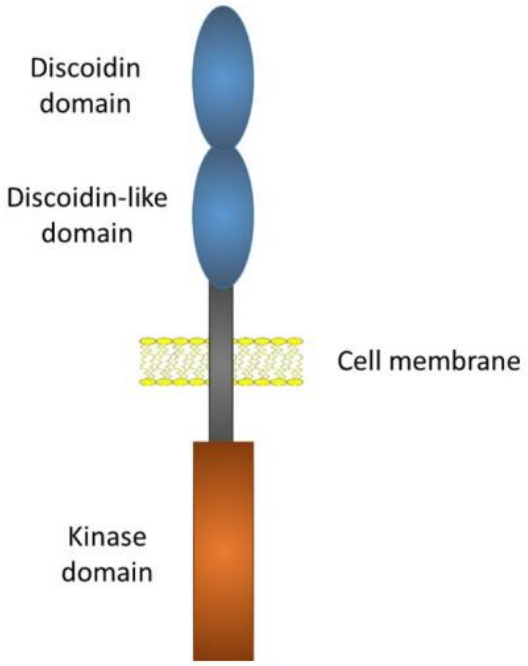
Introduction

Background and Motivation

There are more than 20 types of collagen, each with a characteristic collagenous domain formed by a triple helix of peptides containing Gly-X-Y repeats (where X and Y are frequently prolines and hydroxyprolines). Type I collagen is a major component of the extracellular matrix (ECM) of blood vessel walls in addition to many other connective tissues. Collagen fibrils are known to play an important role in ECM remodeling [1-2], cell-matrix interactions [3-4], and the mechanical properties of the associated tissues [5-8].

The collagen fibril is formed by monomers which assemble and align in a quarter stagger with their neighbors throughout the length of the fibril. This arrangement leads to regions of discrete high and low density which appear as periodic light and dark bands when viewed with transmission electron microscopy (TEM). The length of a single light-dark band is known as the D-period of the collagen fibril structure. The formation of the D-period and overall fibril quality is dictated by various factors. One such factor that can regulate the collagen content and fibril structure within the ECM is the expression of collagen binding proteins (CBP) which can regulate collagen fibrillogenesis. [9-10].

Discoidin domain receptor 1 (DDR1) is a collagen-binding receptor tyrosine kinase that inhibits collagen fibril formation and disrupts the native banded structure of collagen fibers [11-13]. A schematic of discoidin domain receptor (DDR) structure is shown in Figure 1.

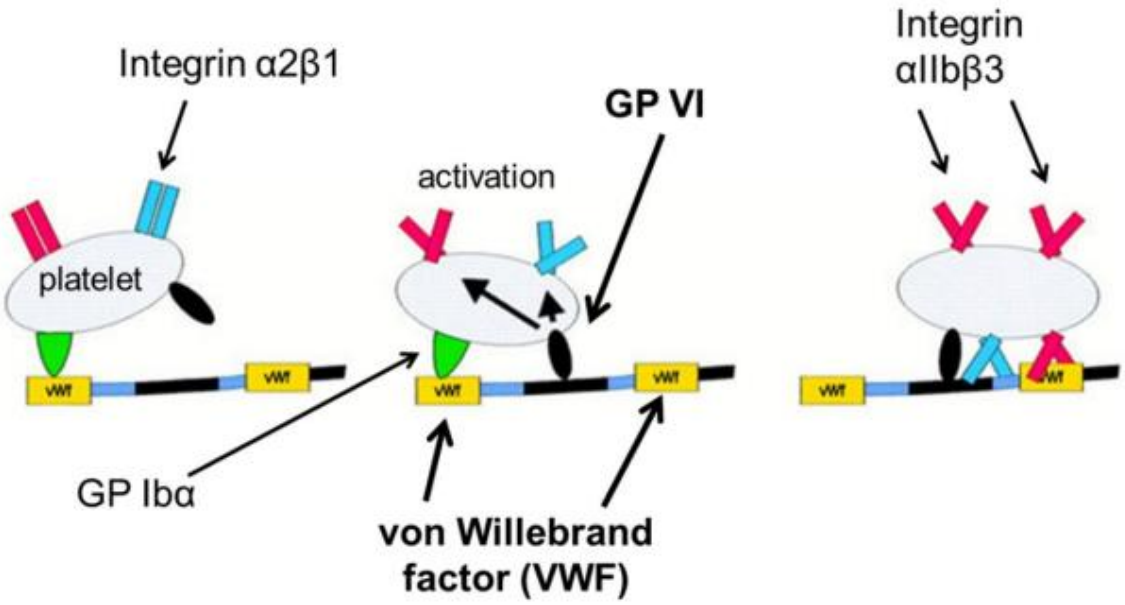


<u>Figure 1</u>: Discoidin Domain Receptor Structure

The extracellular domain (ECD) of DDRs is necessary and sufficient for its binding to collagen. Previous in-vitro studies have elucidated the role of DDRs in regulating collagen deposition, and how they affect the collagen fibril structure[10,12]. Dr. Agarwal's laboratory has recently shown that the adventitia of DDR1 knockout (KO) mice exhibits collagen fibrils with larger diameters, and an increase in the depth of D-periods. DDR1 can thus be used as a modulator of collagen fibril ultrastructure to examine how platelet collagen interactions are affected. This knowledge is important because alterations in collagen fibril structure can affect the functional roles of collagen.

In addition to serving load bearing functions in tissues, collagen fibrils also serve as substrates and ligands for cell-matrix interactions. Cell-collagen interactions occur via cell

surface receptors by binding directly to collagen or indirectly by binding to collagen through matricellular CBPs. Platelet adhesion to collagen is one of the best studied system for cell-collagen interactions (Figure 2).



<u>Figure 2</u>: Adhesion of platelets to collagen fibrils is mediated by GPVI, vWF, and integrin $\alpha_2\beta_1$

Platelet-collagen adhesion is primarily mediated through the binding of platelet receptors, such as glycoprotein VI (GPVI) to collagen and GPIb to collagen-bound von Willebrand factor (VWF) [14-15]. Upon the occurrence of vascular injury, collagen in the subendothelial tissue is exposed to flowing blood. The blood platelets initially interact with collagen via VWF from the blood plasma. VWF has a single, strong binding site to collagen [16-17]. Platelet receptor GP1bα then interacts with the collagen-bound VWF and causes platelets to roll along the injury site [14]. The binding sites for GPIb and collagen are located on different domains of VWF [18]. Another

platelet receptor GPVI also binds to collagen and activates the platelets as they are roll along the exposed collagen [15]. Several GPVI binding sites are present on the collagen fibril.

Platelet-collagen interactions are dependent on the availability and accessibility of these binding sites on the collagen fibril. A change in collagen fibril structure could alter the accessibility of collagen binding sites and impact platelet-collagen interactions. Previous studies have indicated that platelet adhesion is sensitive to collagen fibril structure [19-24]. However, the role of non-platelet derived receptors in modulating platelet-collagen interaction is not well-understood.

Research Significance and Goals

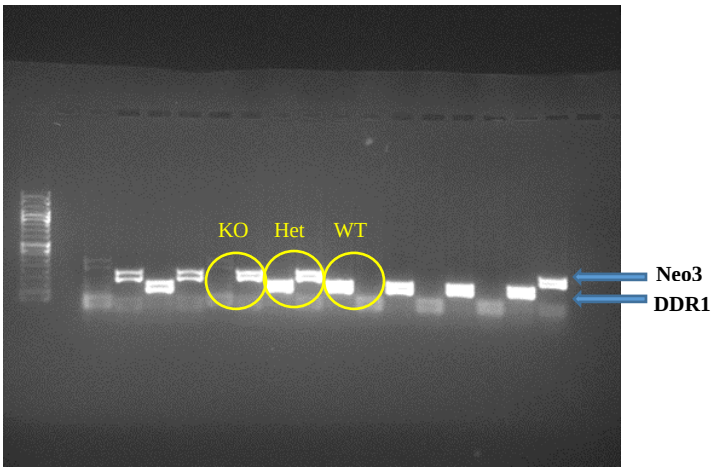
This research project is important to determine how changes in the collagen fibril structure impact platelet-collagen adhesion, and the extent to which altering the number of VWF and GPVI binding sites available on collagen can affect platelet-collagen adhesion. This knowledge is important for pathological conditions, such as atherosclerosis and aneurysms, where collagen is extensively remodeled, which could lead to altered collagen fibril structure. Further insight would be gained through determining platelet adhesion in diseases which are characterized by dysregulation of CBPs like DDR1.

Building upon initial results in Dr. Agarwal's lab to examine how changes in collagen fibril structure observed in the DDR1 KO aortic vessel wall impact platelet adhesion, we hypothesized that platelet adhesion would be altered in DDR1 KO mice in a GPVI or vWF dependent manner.

Methodology

Mice

DDR1 KO mice and their wild-type littermates are available in my advisor's laboratory via an approved IACUC protocol at the Ohio State University. The DDR1 KO mice used in this study have been generated using homologous recombination to silence the DDR1 gene. DDR1 KO mice and their wild type (WT) littermates were obtained through heterozygous breeding at expected Mendelian frequency. Studies were performed on 6-8 month old DDR1 KO mice and their age/gender matched WT littermates. PCR reactions were conducted in order to genotype mice as either WT, DDR1 KO, or heterogeneous. Tail clippings were lysed in Direct PCR lysis buffer and used as a template for PCR. The PCR was conducted using the mouse tail lysates at 95°C for 4 minutes, followed by 30 cycles at 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds. The cycling was followed by a final extension step at 72°C for 7 minutes. The PCR samples were then run on ethidium bromide gels for 50 minutes at 100 V next to a 100 base pair DNA ladder. The resulting gels were analyzed in comparison to the 100 bp ladder to determine the mouse tail lysate samples to be either WT, KO, or heterogeneous (Figure 3).



<u>Figure 3</u>: Gel displaying genotype for WT, KO, and Heterogeneous mice

. The aortas were dissected from the mice within 30 minutes of death to avoid coagulation, and the excised aortas were of the region spanning from the left subclavian artery to the diaphragm. For evaluation of platelet adhesion to collagen fibers, the murine aorta aortic sections were cleaned of perivascular adipose tissue and embedded in Optimal Cutting Temperature (OCT) compound by flash freezing in liquid nitrogen. They were stored frozen at -80^{o}C until use. At the time of usage, 5μm thick sections of the OCT-embedded aorta were cryo-sectioned, mounted on poly-lysine-coated glass microscope slides, washed, and used to perform a static platelet adhesion assay.

Platelet Adhesion Assay and Statistical Analysis

Whole blood (~10 ml) was obtained from healthy adult volunteers in 3.2% sodium citrate tubes via venipuncture in accordance with my advisor's IRB protocol for study of human subjects. Human platelet-rich plasma (PRP) was isolated from the whole blood by centrifugation at 200 x g for immediate use in a static platelet adhesion assay. OCT embedded aortic sections were washed multiple times in PBS before being blocked with 2% bovine serum albumin (BSA). Aortic sections from both DDR1 KO and WT mice were then incubated at room temperature, both with and without VWF or GPVI inhibitors present for 1 hour. The aortic sections were then incubated at room temperature for 30 minutes in 10% PRP under static conditions. (Higher concentrations pf PRP were found to result in saturation pf platelet adhesion). Platelet adhesion to the adventitia of the vessel wall was evaluated using indirect immunofluorescence microscopy after washing off unbound platelets, blocking with 2% BSA, and using anti-GP1b antibody followed by the corresponding fluorescent Alexa Fluor 568 conjugated anti-mouse secondary antibody. Fluorescence images of aortic sections were obtained with a Zeiss Axiovert wide-field

fluorescence microscope using a 20x objective and a TRITC filter. Regions of adventitial collagen were identified using autofluorescence through a DAPI filter.

Quantitative analysis of platelet adhesion to the adventitia was carried out by using ImageJ software to analyze the area of bound platelet particles per unit area of adventitial collagen. The amount of inhibition was determined by subtracting the ratio of inhibited platelet adhesion over non-inhibited platelet adhesion from 1. These platelet adhesion ratios were evaluated for significance between the DDR1 KO and WT mice. Statistical significance in the amount of platelet adhesion and inhibition between DDR1 KO and WT mice was determined using two-tailed t-tests for paired data via Excel. Approximate p-values were given for each t-test with a p-value < 0.05 indicating a statistically significant difference between the data sets.

Results

The fluorescence images of the aortic sections obtained after static platelet adhesion assay were observed to have numerous small fluorescent particles bound to the adventitial region of the aortas, indicative of platelet adhesion (Figure 4). Control samples in which the aorta was not incubated with PRP were observed to be devoid of such fluorescent particles (data not shown). Visual examination of the fluorescent images indicated increased platelet adhesion to the adventitial collagen of the DDR1 KO aortic cross sections as compared to the WT aortic cross sections (Figure 4).

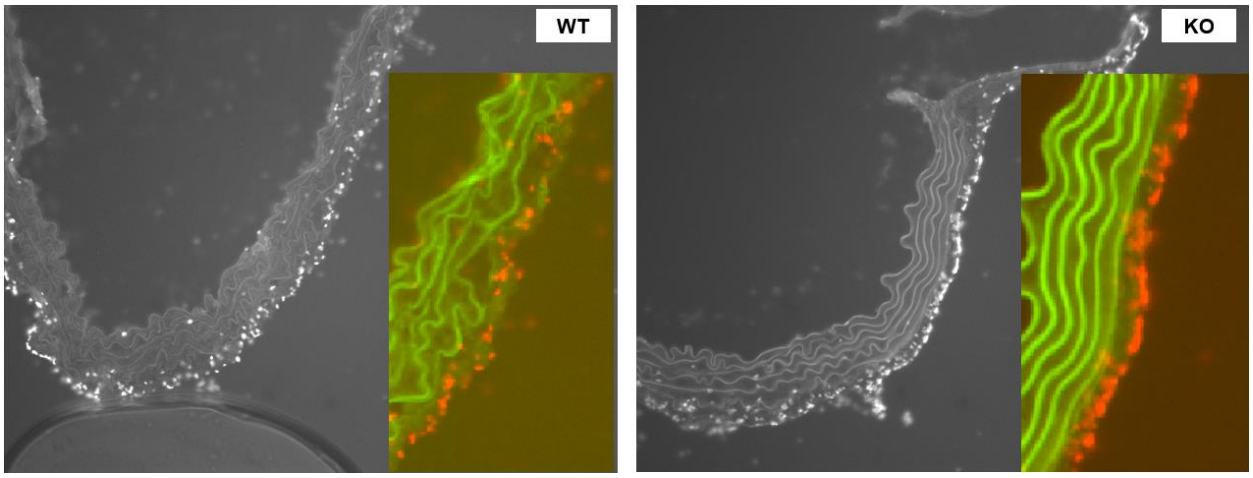
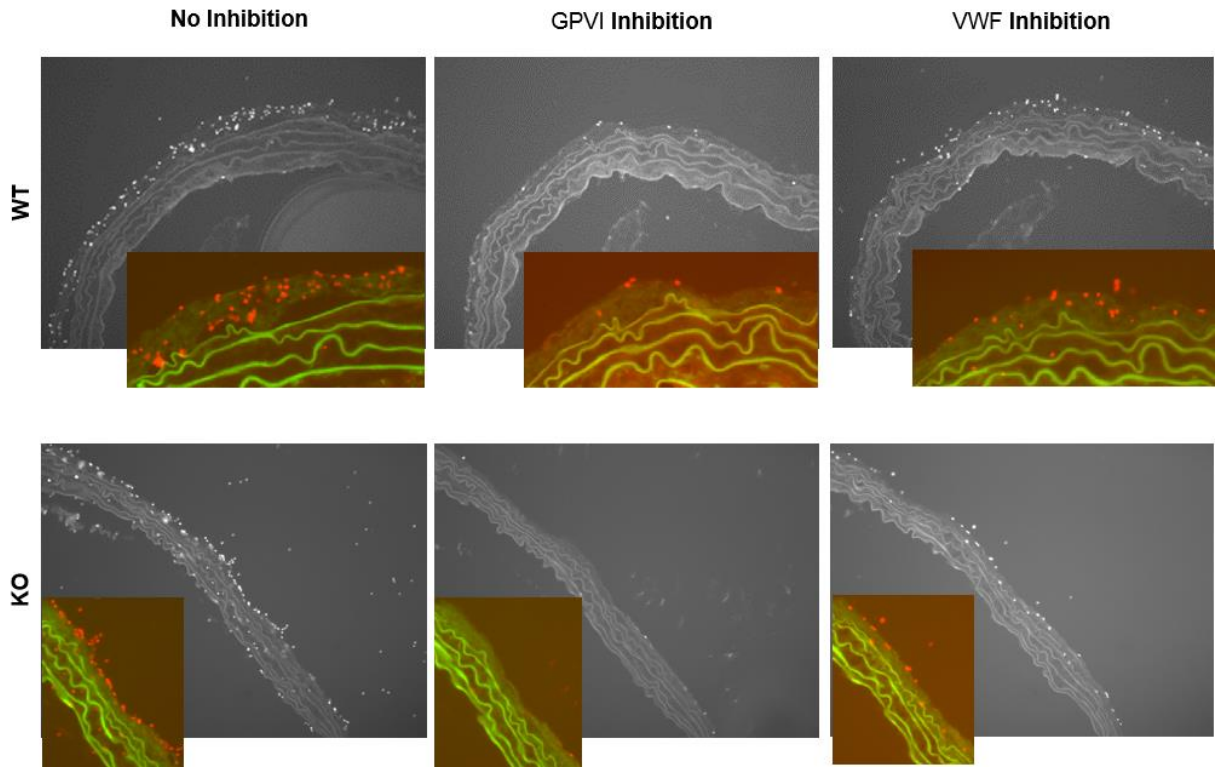


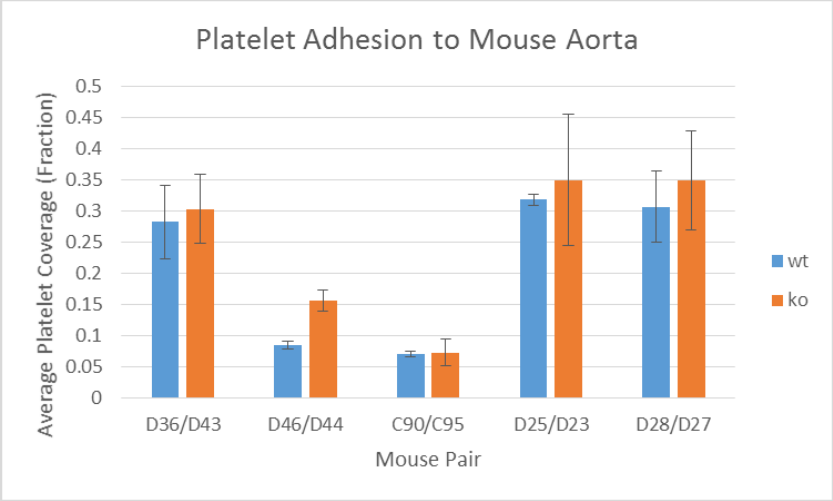
Figure 4: Murine aortic sections were incubated with human PRP. Platelet adhesion to the adventitia was determined by analyzing the area of bound platelet particles (red) per unit area of adventitial collagen (green). D28/WT (left), D27/KO (right)

The aortic sections in which the PRP incubation was preceded by the incubation of GPVI or VWF inhibitors displayed a significant decrease in the amount of fluorescent particles present as compared to the non-inhibited sample for any given mouse (Figure 5). This observation was consistent between both the WT and DDR1 KO mice; however, the degree of inhibition appeared to be to varying degrees between WT and DDR1 KO mice within any given pair.



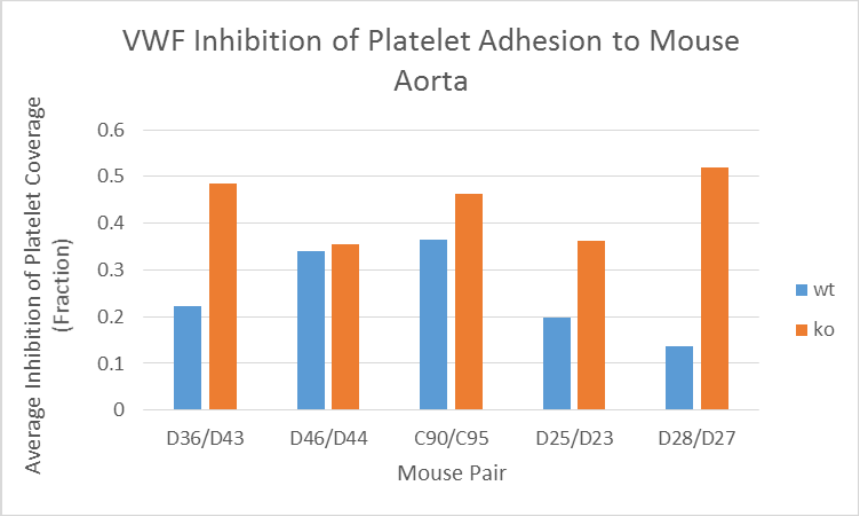
<u>Figure 5</u>: Platelet adhesion to murine aortic sections in the presence of GPVI and vWF-collagen inhibitor. Degree of inhibition was determined as (1-AI/Ao), where AI=adhesion in the presence of inhibitor and Ao=non-inhibited platelet adhesion - D46 (WT), D44 (KO).

In order to determine if the difference in platelet adhesion and degree of inhibition between WT and DDR1 KO mice was due to changes in the collagen fibril ultrastructure, quantitative analysis of platelet adhesion to the adventitia was carried out by analyzing the area of bound platelet particles per unit area of adventitial collagen. This would account for the differences in the adventitial thickness between the various mice. The analysis determined that platelet adhesion normalized to the adventitial collagen was ~1.2X greater for the aortic cross sections of DDR1 KO mice than that of their WT mouse pair counterpart (Figure 6; $p<0.05$; n=5 mouse pairs) despite donor variations.

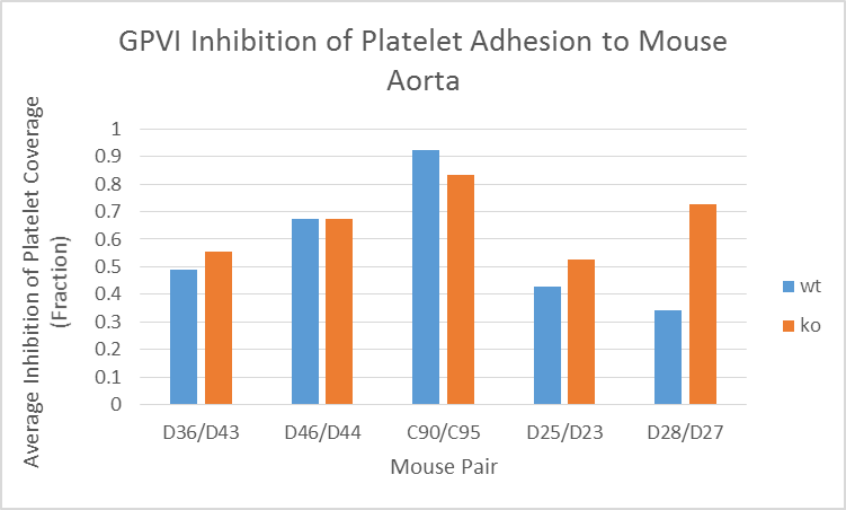


<u>Figure 6</u>: Platelet adhesion normalized to the adventitial collagen was ~1.2X greater for the aortic cross sections of DDR1 KO mice than that of their WT mouse pair counterpart ($p<0.05$; n=5 mouse pairs).

Quantitative analysis of the degree of inhibition was determined by subtracting the ratio of inhibited platelet adhesion (either in the presence of GPVI or VWF inhibitor) over non-inhibited platelet adhesion from 1 for each mouse sample. The analysis determined that the degree to which platelet adhesion was inhibited via VWF was ~1.8X greater for the DDR1 KO mice than it was for the WT counterparts (Figure 7; $p<0.05$; n=5 mouse pairs). Analysis of the GPVI inhibition indicated that there was a slight trend supporting that DDR1 KO mice were inhibited to a greater degree than WT mice in 4 of the 5 mouse pairs, but the differences were not found to be statistically significant (Figure 8; $p>0.05$; n=5 mouse pairs).



<u>Figure 7</u>: Analysis of platelet adhesion inhibition between WT/KO mouse pairs via VWF inhibition showed that the degree to which platelet adhesion was inhibited via VWF was ~1.7X greater for the DDR1 KO mice than it was for the WT counterparts ($p<0.05$; n=5 mouse pairs).



<u>Figure 8</u>: Analysis of platelet adhesion inhibition between WT/KO mouse pairs via GPVI inhibition indicated that although there was a trend supporting that DDR1 KO mice were inhibited to a greater degree than WT mice in 4 of the 5 mouse pairs, the differences were not found to be statistically significant ($p>0.05$; n=5 mouse pairs).

Discussion

Our results supported our hypothesis in that changes in collagen fibril structure as observed in the DDR1 KO aortic vessel wall impact platelet-adhesion. Additionally, our results show that platelet adhesion is modulated primarily by GPVI in static-adhesion assays as platelet adhesion was almost entirely inhibited by inhibiting GPVI. Quantitative analysis of platelet adhesion indicated that the number of GPVI binding sites available on collagen fibrils was more in the DDR1 KO mice, which resulted in an increase in platelet-adhesion to collagen fibrils (Figure 9).

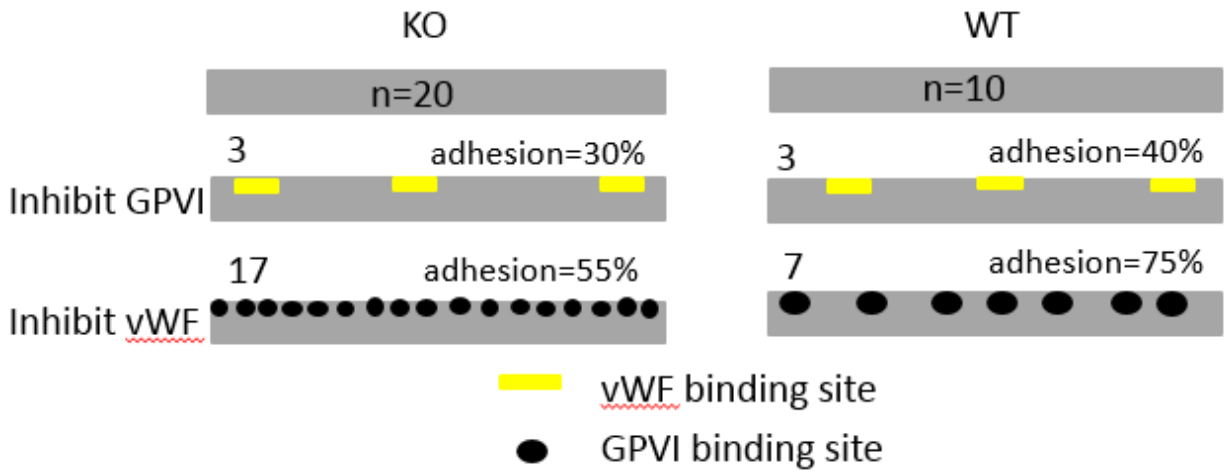


Figure 9: Schematic illustrating availability of platelet binding sites on collagen fibrils in DDR1 KO and WT mice based on our experimental results. (i) the total number (n) of platelet binding sites exposed on collagen are higher in DDR1 KO resulting in increased platelet adhesion. (ii) The number of GPVI sites are higher than the vWF binding sites in both genotypes as inhibiting vWF results in less inhibition and more adhesion compared to GPVI.

All experiments in this study were carried out under static conditions. These conditions most closely mirror blood vessels with low shear rates, such as veins; however, these conditions do not adequately replicate arterial flow conditions in vivo. In future studies, this will be

accomplished in experiments using a dynamic platelet adhesion assay to more closely imitate the wider range of shear rates that arterial blood flow is subject to in vivo. In addition to platelet-collagen interactions, other types of cells may also be affected by alterations to the collagen fibril. As such, progression or remission of a disease may depend on cell-collagen interactions with irregular collagen fibrils. Future experiments will span into additional biological phenomena beyond platelet-collagen adhesion that are influenced by DDR1 deficiency and altered collagen ultrastructure. Beyond DDR1, other CBPs could also serve as modulators of collagen fibril structure and a multifactorial approach may need to be developed to diagnose and treat pathological ECM remodeling.

Conclusion

In summary, DDR1 KO mice have increased number of bound platelet aggregates as compared to WT mice. When GPVI platelet binding sites were inhibited, there was a trend supporting that DDR1 KO mice are inhibited to a greater degree than their WT littermates, but the difference was not found to be statistically significant. When VWF platelet binding sites were inhibited, DDR1 KO mice were inhibited to a greater degree than the WT mice. From these findings, we are able to conclude that changes in collagen fibril ultrastructure impact platelet-collagen adhesion by altering the number of GPVI binding sites available on collagen fibrils, resulting in increased platelet adhesion in DDR1 KO mice. Additionally, we are able to conclude that the number of GPVI binding sites are greater than the number of VWF binding sites along the collagen fibril in both genotypes, as inhibiting VWF results in less inhibition compared to GPVI. These findings may be important for understanding certain pathological conditions, such as atherosclerosis and aneurysms, where collagen is extensively remodeled, and could lead to altered collagen fibril structure and thrombogenic events.

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